Equol-stimulated mitochondrial reactive oxygen species activate eNOS and redox signaling in endothelial cells: Roles for F-actin and GPR30

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Online Data Supplement - Extended Materials and Methods

Materials

Equol was from Apin Chemicals Ltd (UK); MnTMPyP and AG1478 from Calbiochem (USA); L-NAME, IBMX, SOD, PEG-SOD, PEG-CAT, rotenone and pertussis toxin from Sigma-Aldrich (UK). Monoclonal antibodies against eNOS and secondary antibodies were from Santa Cruz Biotechnology (USA), α-tubulin from Chemicon (USA), antibodies against eNOS-Ser1177, dually phosphorylated (threonine 183 and tyrosine 185) ERK1/2 and Akt-Ser473 from Cell Signaling (UK). ECL reagent was from Amersham (UK); MitoSox Red from Invitrogen Molecular Probes (USA); Rhodamine-phalloidin and Hoechst reagent from Fluka (UK); cGMP ELISA from Cayman Chemicals (USA).

Human Umbilical Vein Endothelial Cell Culture

Umbilical cords were obtained with informed patient consent and approval from the St. Thomas' Hospital Ethics Research Committee. All experiments were performed with passage 3 HUVEC, and an endothelial phenotype was confirmed by a characteristic cobblestone morphology and positive immunostaining for von Willebrand factor (data not shown).[1-3]

Immunoblotting

HUVEC (passage 3) were equilibrated in low serum (1% FCS) M199, preincubated in Krebs buffer containing L-arginine (100μM) for 30 min and then treated for 2 min with equol (100nmol/L) or vehicle (0.01% DMSO). In other experiments, cells were pretreated for 30 min in the absence or presence of SOD (200U/mL), PSOD (50U/mL), PCAT (200U/mL), rotenone (2μmol/L), pertussis toxin (100ng/mL), AG-1478 (5μmol/L, EGFR tyrosine kinase inhibitor) or 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4-d] pyrimidine (10μmol/L, PP2, c-Src kinase inhibitor) and then challenged acutely for 2 min with equol (100nmol/L). Reactions were stopped with ice-cold PBS, cell lysates separated by SDS-PAGE, probed with specific antibodies, protein bands detected by ECL and densitometric analyses performed using Image J software (National Institute of Health, USA).[2]

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Quantitative RT-PCR

HUVEC were stimulated with equol (100 nmol/L) for 6h, cells lysed and RNA purified using the Macherey-Nagel RNA isolation kit, quantified and reverse-transcribed using a QuantiTect RT kit (Qiagen).[4] eNOS gene expression was analyzed using a quantitative RT-PCR system (Corbett Rotor-gene) and mRNA levels were normalized to the geometric mean of three stable reference genes (ribosomal protein L13a (RPL13A), succinate dehydrogenase (SDHA), β2-microglobulin (β2M).[5,6] Primer sequences were:

eNOS: 5'-GCATCACCAGGAAGAAGACCT-3' and 5'-TTCACTCGCTTCGCCACAC-3'
RPL13A: 5'-GAGGCCCCTACCACTTCC-3' and 5'-AACACCTTGAGACGGTCCAG-3'
SDHA: 5'-AGAAGCCCTTTGAGGAGCA-3' and 5'-CGATTACGGGTCTATATTCCAGA-3'
β2M: 5'-TTCTGGCCTGGAGGCTATC-3' and 5'-TCAGGAAATTTGACTTTCCATTC-3'

Intracellular cGMP Accumulation as an Index of NO Production

Confluent cell monolayers were preincubated for 30min with Krebs buffer containing L-arginine (100μmol/L) and the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 0.5 mmol/L) in the absence or presence of L-NAME (100μmol/L) or rotenone (2μmol/L).[1-3] Cells were then stimulated acutely for 2min with equol (100nM) or vehicle (Veh, 0.01% DMSO) in the presence of IBMX (0.5mM) and L-arginine (100μmol/L) and continued absence or presence of L-NAME (100μmol/L) or rotenone (2μmol/L). Basal and stimulated intracellular cGMP levels were determined by ELISA, with inhibition of cGMP accumulation by L-NAME serving as an index of NO production.[1-3]

References

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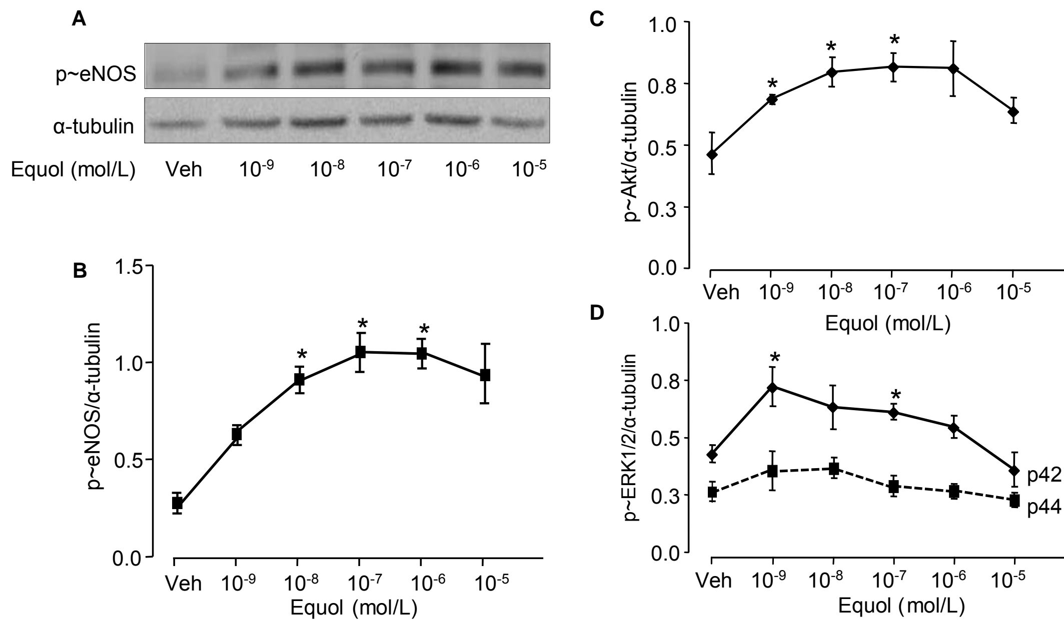

Figure S1. Concentration-dependent activation of eNOS and upstream kinases by the isoflavone equol. HUVEC were equilibrated in low serum (1% FCS) M199 for 4 h and then incubated in Krebs buffer containing L-arginine (100µmol/L) and stimulated for 2 min with equol (10^{-9}–10^{-5} M) or vehicle (Veh, 0.01% DMSO). Cell lysates were immunoblotted for p~eNOS, p~Akt and p~ERK1/2. (***A***) Representative immunoblot of eNOS phosphorylation. (***B-D***) Densitometric analyses of p~eNOS, p~Akt and p~ERK1/2 in HUVEC stimulated acutely with equol vs. vehicle. Mean ± S.E.M. of values in cultures from 3-4 different donors, *P<0.05 relative to vehicle.

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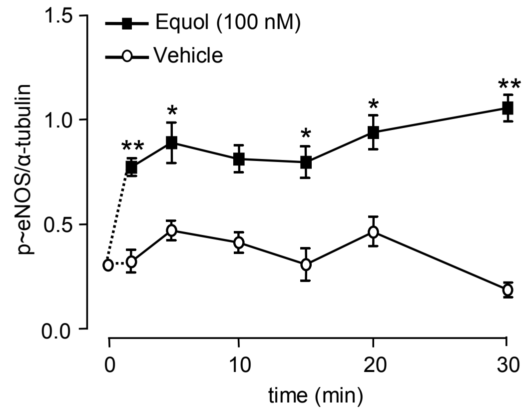

Figure S2. Time-dependent activation of eNOS phosphorylation by the isoflavone equol. HUVEC were equilibrated in low serum (1% FCS) M199 for 4 h and then incubated in Krebs buffer containing L-arginine (100 µmol/L) and stimulated for 2 – 30 min with equol (100 nmol/L) or vehicle (Veh, 0.01% DMSO). Cell lysates were immunoblotted for phosphorylated eNOS-Ser1177 and analyzed by densitometry relative to α-tubulin. Mean ± S.E.M. of values in cultures from 3-4 different donors, **P*<0.05, ***P*<0.01 relative to vehicle.

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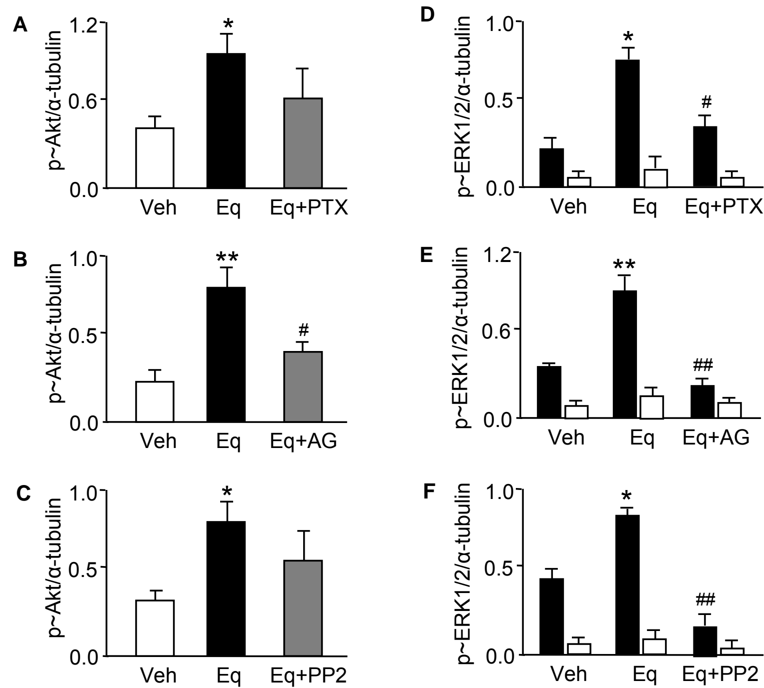

Figure S3. Densitometric analysis of equol stimulated Akt and ERK1/2 phosphorylation in fetal endothelial cells - effects of inhibitors of G-proteins, EGFR kinase and c-Src kinase. HUVEC were pre-equilibrated in low serum (1% FCS) M199 for 4 h and then pre-incubated for 30 min with the G-protein inhibitor (pertussis toxin, PTX, 100 ng/mL), EGFR kinase inhibitor (AG-1478, AG, 5μmol/L) or Src kinase inhibitor (PP2, 10 μmol/L) prior to acute stimulation with equol (Eq 100 nmol/L) for 2 min in the absence or presence the inhibitors. Membranes were immunoblotted for p~Akt (panels A-C) and p~ERK1/2 (panels D-F) vs. α-tubulin. Mean ± S.E.M of measurements in different cell cultures obtained from 4 different donors, *P<0.05, P<0.01 vs. vehicle (Veh, 0.01% DMSO) and #P<0.05, ##P<0.01 vs. equol in the absence of inhibitors.

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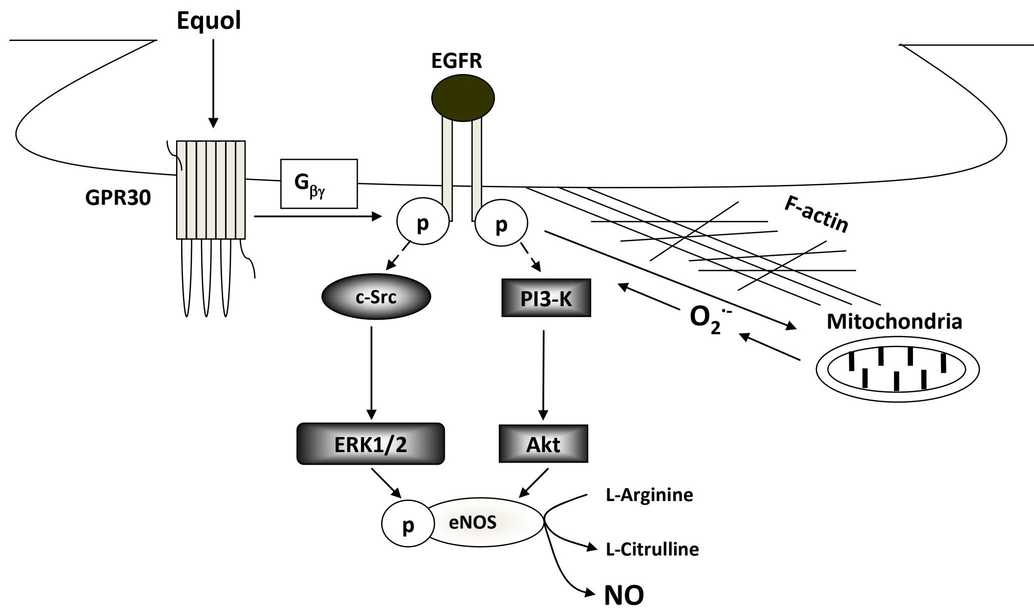

Figure S4. Proposed mechanisms underlying acute activation of eNOS by equol and other dietary isoflavones. Equol and other dietary isoflavones stimulate kinase activation and subsequent eNOS phosphorylation and NO production via mitochondria-derived ROS. Kinase activation may be dependent on GPR30/epidermal growth factor receptor (EGFR) transactivation as previously described.[21,24] EGFR activation leads to ERK1/2 activation via c-Src and mitochondrial-ROS potentiated Akt activation via PI-3K. Notably, inhibition of mitochondrial ROS generation with rotenone or depolymerization of F-actin abrogates equol stimulated activation of Akt, eNOS and NO production in human endothelial cells.

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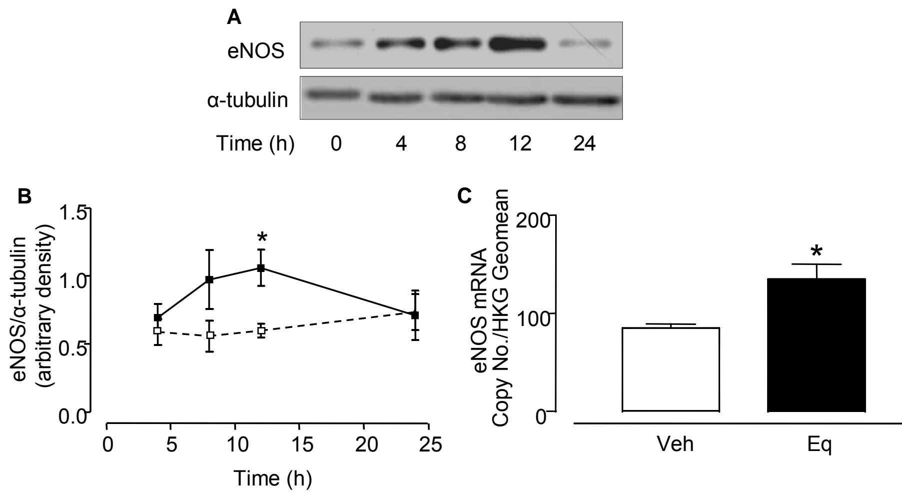

Figure S5. Equol upregulates eNOS protein and mRNA expression in fetal endothelial cells. HUVEC were pre-incubated in low serum (1% FCS) M199 for 4 h and then treated with M199 (1% FCS) containing equol (Eq, 100 nmol/L) or vehicle (Veh, 0.01% DMSO) for 4 – 24 h. (**A-B**), effects of equol on eNOS protein expression in a representative immunoblot and densitometric analysis of eNOS/α-tubulin ratio. (**C**) eNOS mRNA expression after 6 h treatment with equol (Eq, 100 nmol/L, filled squares) *vs.* vehicle (Veh, 0.01% DMSO, unfilled squares), expressed as mean copy number relative to the Geomean of three house-keeper genes (see Supplemental Materials and Methods). Means ± S.E.M., n = 5-6 different cell cultures from 5-6 different donors, *$P<0.05$, *vs*. vehicle.

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